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# Sequence specificity of the non-natural pyrido[2,3-*d*]pyrimidine nucleoside in triple helix formation

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## ABSTRACT

**The non-natural pyrido[2,3-*d*]pyrimidine nucleoside F, which pairs preferentially with guanine (G) and adenine (A) within double-helical DNA, recognizes with high selectivity AT base pairs within triple-helical complexes. These observations suggest that F may exist in different tautomeric forms within double-helical and triple-helical complexes. Analysis of the base stacking properties of this extended ring system using two oligodeoxyribonucleotides containing terminal thymines and/or pyrido[2,3-*d*]pyrimidines bound to adjacent sites showed a decrease in free energy of binding in a triple-helical complex in the order (5′–3′) TT > FT > TF > FF.**

## INTRODUCTION

Oligonucleotide-directed formation of local triple-helical structures may serve as a powerful method to target single sites within large sequences of double-helical DNA (1, 2). There are two major triple-helical structures through which oligodeoxyribonucleotides bind purine tracts in the major groove of double-helical DNA. Pyrimidine-rich oligodeoxyribonucleotides bind parallel to the Watson–Crick purine strand through formation of Hoogsteen hydrogen bonds between thymine (T) and adenine–thymine (AT) base pairs (T·AT) and between protonated cytosine (C+) and guanine–cytosine (GC) base pairs (C+GC) (1–4), whereas purine-rich oligodeoxyribonucleotides bind antiparallel to the Watson–Crick purine strand through reverse Hoogsteen hydrogen bond formation between G and GC base pairs and A or T and AT base pairs (5–8). The discovery of other natural and non-natural base triplets (9–18) and the use of alternate strand triple-helix formation (19–23) has increased the number of double-helical sequences that can be recognized beyond simple purine tracts. However, a general solution for specific recognition of all *four* natural bases through triple-helix formation has still not been achieved. The design criteria for heterocycles that can selectively recognize double-helical DNA include (i) appropriate positioning of hydrogen bond donors and acceptors for pairing with the edges of AT and GC Watson–Crick base pairs, (ii) a non-disruptive backbone geometry of the novel nucleotide, and (iii) optimal base stacking within the third strand.

It has previously been reported that the non-natural pyrido[2,3-*d*]pyrimidine nucleoside F pairs with G and A within double-helical DNA (24). One explanation is that a single tautomeric form of F forms a Watson–Crick base pair with G and, through a backbone shift, a wobble base pair with A (24). An alternative explanation is that F exists in different tautomeric forms $F_1$ and $F_2$, whose equilibrium is influenced by the base in the opposing DNA strand (Figure 1a). The heterocycle F is structurally related to the natural pyrimidine bases and should therefore be able to maintain a correct backbone geometry within the pyrimidine–purine–pyrimidine triple-helix. Tautomer $F_1$ carrying the proton at N-8 resembles a C base analog, which may allow recognition of GC and/or CG base pairs within the pyrimidine triple-helical motif (Figures 2, 3a). Tautomer $F_2$ carrying the proton at N-1 corresponds to a T analog, which should recognize AT base pairs within a triple-helical complex (Figures 2, 3b). The following report presents experiments designed to identify the base specificity and binding affinity of the non-natural pyrido[2,3-*d*]pyrimidine nucleoside F within triple-helical complexes.

## MATERIALS AND METHODS

### General

The 5-methyl-2′-deoxycytidine (d<u>C</u>) phosphoramidite was purchased from Glen Research. All other phosphoramidites and chemicals for DNA synthesis were obtained from Applied Biosystems Inc. All enzymes were purchased from Boehringer-Mannheim, New England Biolabs or Sigma. Restriction endonucleases were used according to the supplier's recommended protocol in the activity buffer provided. The Sequenase™ DNA sequencing kit (Version 2.0) was obtained from United States Biochemical Inc. Deoxynucleoside triphosphates (100 mM solutions), calf thymus DNA, NICK™-columns and NAP-5 Sephadex columns were purchased from Pharmacia LKB. Glycogen was obtained from Boehringer-Mannheim as a 20 mg/ml aqueous solution. The radiolabeled triphosphates [$\alpha$-$^{32}$P]dGTP (> 3000 Ci/mmol), [$\gamma$-$^{32}$P]ATP (> 5000 Ci/mmol) and [$\alpha$-$^{35}$S]dATP (> 1000 Ci/mmol) were obtained from Amersham. All other chemicals were of analytical or HPLC grade. Standard molecular biological methods were used, if not mentioned otherwise (25, 26). Thermal denaturation

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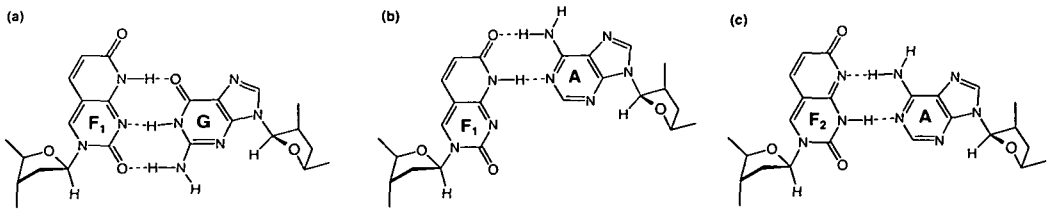


**Figure 1.** Possible hydrogen bond formation between pyrido[2,3-*d*]pyrimidine F and the natural bases G and A. $F_1$ and $F_2$ refer to different possible tautomers.

profiles were obtained on a Hewlett-Packard 8452A diode array spectrophotometer.

### Synthesis of oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer using standard solid-phase β-cyanoethyl phosphoramidite chemistry (26, 27). The pyridopyrimidine heterocycle F was prepared as previously reported in the literature (28) and incorporated either within or at the 5′-end of an oligodeoxyribonucleotide during a regular cycle or at the 3′-end of an oligodeoxyribonucleotide via the 5′-*O*-DMT−thymidine−EDTA−triethylester-3′-succinyl controlled pore glass (29). The thymidine−EDTA nucleoside analog T* was prepared according to published procedures and incorporated at the 5′ end (30). Unmodified oligodeoxyribonucleotides were deprotected in concentrated ammonium hydroxide at 55°C for 24 hours. Oligodeoxyribonucleotides containing the nucleoside analog T* were treated with 0.1 N NaOH (1.5 ml) at 55°C for 24 hours and the resulting solutions were neutralized with glacial acetic acid. The resulting mixture was applied to a column of Sephadex G10-120 and eluted with water. The crude oligodeoxyribonucleotides were lyophilized and then purified by electrophoresis on 20% denaturing polyacrylamide gels (19:1 monomer/bis). The major UV-absorbing bands were excised, crushed and eluted (0.5 M NaCl, 0.5 ×TE, 37°C, 36 h). After filtration through a Centrex filter (0.45 μm) the oligodeoxyribonucleotides were desalted on Pharmacia NAP-5 Sephadex columns and then exhaustively dialyzed against water. The concentrations of the oligodeoxyribonucleotides were determined by UV measurements at 260 nm on a Hewlett-Packard 8452A diode array spectrophotometer, using the following molar extinction coefficients: 15400 (dA), 11700 (dG), 5700 (dC), 8800 (T and T*), and 15000 (F) $cm^{-1}$ $M^{-1}$. The oligodeoxyribonucleotide solutions were then divided in aliquots, lyophilized to dryness, and stored at −78 °C.

### HPLC analyses

Analytical HPLC was performed with a Hewlett-Packard 1090 Liquid Chromatograph using a reverse phase VYDAC 201HS54 4.6 mm×25 cm 5 micron C18 column. The purified oligodeoxyribonucleotides (3 nmol) were digested simultaneously with snake venom phosphodiesterase (3 μl, 2.4 μg/μl) and calf intestine alkaline phosphatase (3 μl, 1 U/μl) in 50 mM Tris−HCl (pH 8.1), 100 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 3h, filtered through a 0.45 μm Nylon-66 syringe filter (Rainin) and lyophilized. The sample was dissolved in 10 μl water, and an aliquot of the solution was injected onto the C18 reverse phase column. The products were eluted with 20 mM triethylammonium acetate and 1 mM EDTA with a gradient

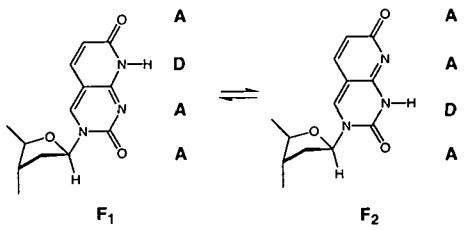


**Figure 2.** Tautomeric forms of F.

of 2.5−50% acetonitrile and detected by UV absorption at 260 and 330 nm. Comparison with standard solutions of dA, dG, dC, T and dF confirmed the composition of the oligodeoxyribonucleotides.

### DNA manipulations

Plasmid DNA (pPG1133 or pMD5152) was linearized with *Eco*RI, followed by treatment with [α-$^{32}$P]dATP and [α-$^{32}$P]TTP in the presence of Sequenase™ (version 2.0). Unincorporated radiolabeled nucleotide triphosphates were removed by filtration of the reaction mixture through a NICK™-column. The labeled DNA was precipitated and digested with *Pvu*II in the case of pPG1133 or *Nde*I in the case of pMD5152. The resulting 3′-$^{32}$P-end labeled fragments were purified by 5% nondenaturing polyacrylamide gel electrophoresis. Gel bands were visualized by autoradiography, excised and crushed, and eluted with 250 mM NaCl, 10 mM Tris, pH 8.0 at 37°C for 24 h. The resulting mixture was filtered through a 0.45 μm Centrex filter, and the eluted DNA was ethanol precipitated and dried *in vacuo*. The pellet was resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), extracted 3−5 times with phenol, and twice with 24:1 chloroform/isoamyl alcohol, and ethanol precipitated. The DNA pellet was washed with 70% ethanol, dried *in vacuo*, and resuspended in 25 mM Tris−acetate, 100 mM NaCl, pH 7.0 and stored at −78°C.

### Quantitative affinity cleavage titrations

In a typical quantitative affinity cleavage titration experiment, involving eighteen data lanes and one control lane per gel, a stock solution containing labeled target DNA in association buffer was prepared by mixing 50 mM Bis-Tris−HCl buffer at pH 7.0, 500 mM NaCl, 1.25 mM spermine hydrochloride, calf thymus DNA at a concentration of 2.0 mM in base pairs, approximately 400,000 cpm of 3′-end labeled target DNA, and enough water to bring to a total volume of 665 μl. The stock solution was then distributed among nineteen 1.5 ml microcentrifuge tubes in 35

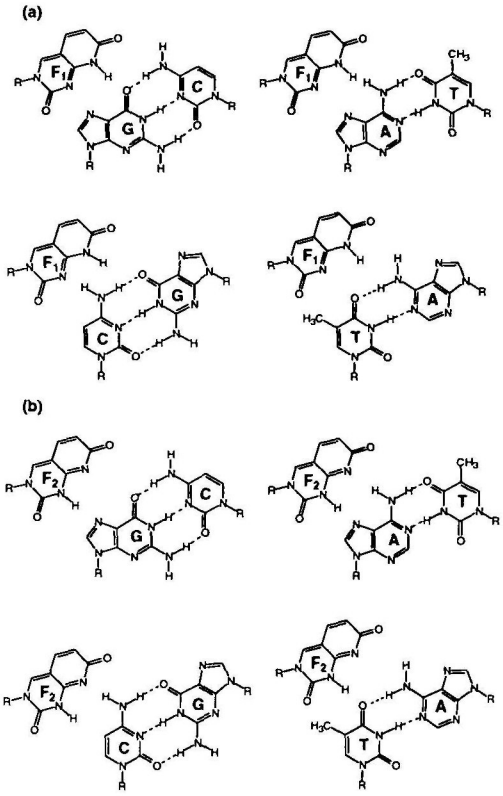

**Figure 3.** Two dimensional models of base triplets containing either (**a**) $F_1$ or (**b**) $F_2$ opposite all four Watson–Crick base pairs.

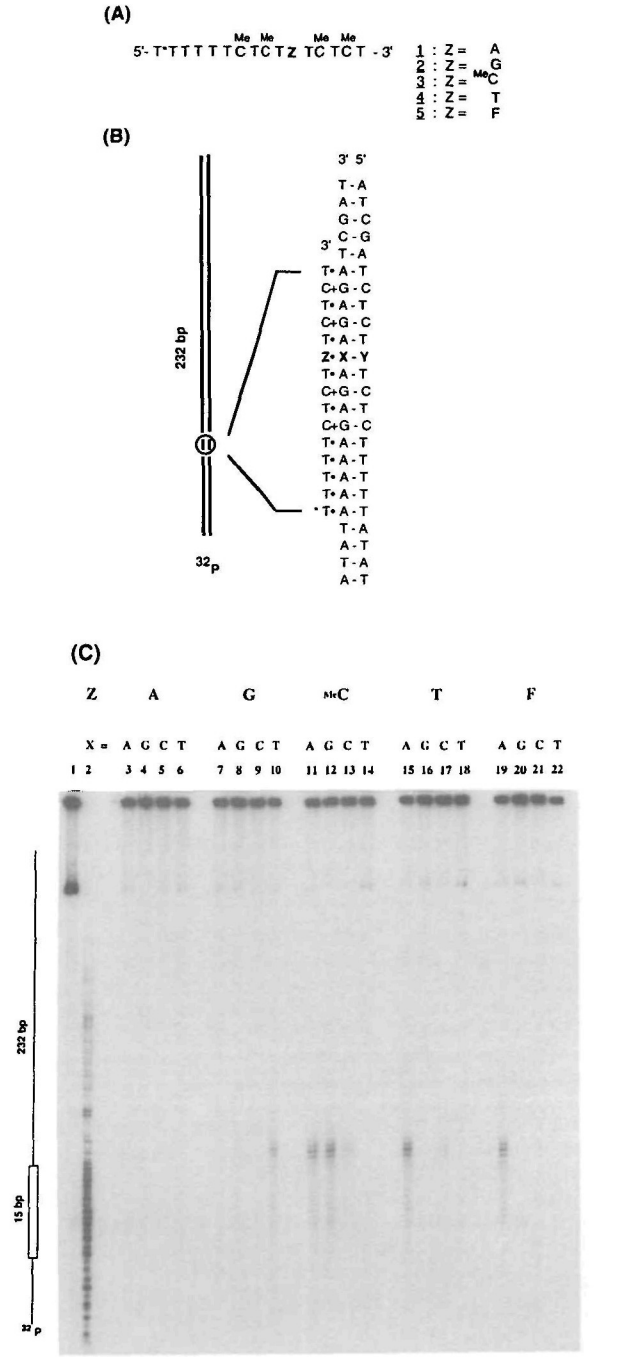

**Figure 4.** Qualitative analysis comparing the relative stabilities of the 16 natural base triplets with the four nonnatural base triplets F·AT, F·GC, F·CG, and F·TA. (**A**) Sequences of oligonucleotide-EDTA **1**–**5**; (**B**) Schematic drawing of the triple-helix complex between the bound oligonucleotide-EDTA·Fe(II) and a single site containing all four base pairs (X-Y); (**C**) Autoradiogram of a 8% denaturing polyacrylamide gel: the cleavage reactions containing the oligonucleotide-EDTA (1 $\mu$M) and Fe(II) (25 $\mu$m) with the $^{32}P$ labeled restriction fragment in association buffer (50 mM Tris–acetate, pH 7.0, 100 mM NaCl, 1 mM spermine, 100 $\mu$M bp calf thymus DNA) were allowed to equilibrate at room temperature for 24 h. The cleavage reactions were initiated by addition of DTT (4 mM) and allowed to proceed for 6 h at room temperature. The reactions were stopped by precipitation with ethanol and the cleavage products were analyzed by gel electrophoresis. Lane 1: control showing intact restriction fragment; lane 2: products of chemical sequencing reaction; lane 3–18: DNA cleavage products produced by oligonucleotide-EDTA·Fe(II) [Z=A (lanes 3–6), Z=G (lanes 7–10), Z=$^{Me}$C lanes (11–14), Z=T (lanes 15–18), Z=F (lanes 19–22)].

$\mu$l aliquots. A dried pellet of the oligodeoxyribonucleotide–EDTA (5 nmol) was dissolved in aqueous solution to produce a 100 $\mu$M solution of oligodeoxyribonucleotide and was diluted serially. To each reaction tube was added 5 $\mu$l of oligodeoxyribonucleotide at the appropriate concentration and 5 $\mu$l of a 75 $\mu$M solution of $Fe(NH_4)_2(SO_4)_2 \cdot 36H_2O$. The oligodeoxyribonucleotide–EDTA·Fe(II) and the DNA were allowed to equilibrate for 24 h at 24°C. The cleavage reactions were initiated by the addition of 5 $\mu$l of a 40 mM DTT solution to each tube. The reactions were incubated for 6 h at 24°C. Final reaction conditions for the affinity cleaving reactions to determine base specificity in 50 $\mu$l association buffer were 10 mM Bis-Tris–HCl at pH 7.0, 100 mM NaCl, 0.25 mM spermine, 0.1 mM base pairs calf thymus DNA, 4 mM DTT, and approximately 21,000 cpm labeled duplex. Final reaction conditions for the affinity cleaving reactions to determine cooperative interactions in 50 $\mu$l association buffer were 25 mM Tris–acetate at pH 7.0, 10 mM NaCl, 1 mM spermine, 0.1 mM base pairs calf thymus DNA, 4 mM DTT, and approximately 21,000 cpm labeled duplex. The cleavage reactions were quenched by the addition of glycogen to a final concentration of 70 $\mu$g/ml, NaOAc to a final concentration of 280 mM (pH 5.2), and $MgCl_2$ to a final concentration of 10 mM, The DNA was precipitated with 2.5 volumes cold ethanol, isolated by centrifugation and subsequently dissolved in 20 $\mu$l distilled water. The solutions were frozen and the solvent was removed by lyophilization. The DNA in each tube was resuspended in 5 $\mu$l formamide–TBE loading buffer, and the solutions were

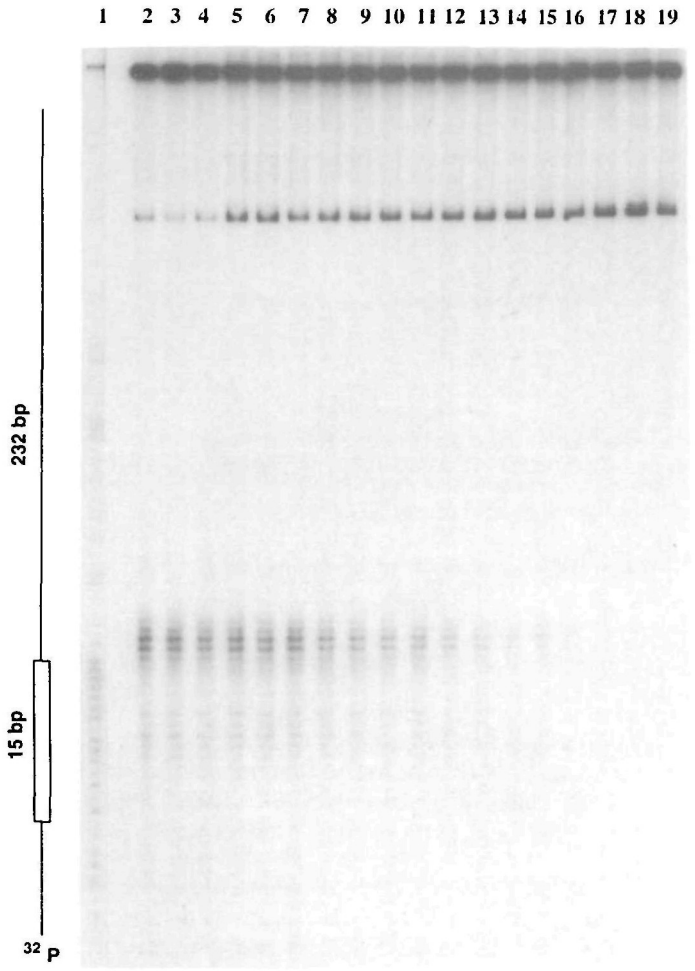


**Figure 5.** Autoradiogram of an 8% denaturing polyacrylamide gel of reaction products from affinity cleavage reactions with oligonucleotide **5** (Z=F) and a $^{32}$P-labeled restriction fragment containing the target site shown in Figure 4 where XY=AT. The reactions were performed at 24°C in 10 mM Bis-Tris. HCl pH 7.0, 100 mM $Na^+$, 250 $\mu$M spermine tetrahydrochloride. 100 $\mu$M bp calf thymus DNA. Lane 1: products of chemical sequencing reaction; lanes 2–19: DNA affinity cleavage reaction products produced by **5** at various concentrations: 10 $\mu$M (lane 2); 8 $\mu$M (lane 3); 4$\mu$M (lane 4); 2 $\mu$M (lane 5); 1$\mu$M (lane 6); 800 nM (lane 7); 400 nM (lane 8); 200 nM (lane 9); 100 nM (lane 10); 80 nM (lane 11); 40 nM (lane 12); 20 nM (lane 13); 10 nM (lane 14); 8 nM (lane 15); 4 nM (lane 16); 2 nM (lane 17); 1 nM (lane 18); no oligonucleotide (lane 19).

transferred to new tubes, assayed for specific activity by scintillation counting, and if necessary diluted to 3000–4000 cpm/$\mu$l with more formamide–TBE loading buffer. The DNA was denatured at 90°C for 5 min and 5 $\mu$l of each sample were loaded onto an 8% denaturing polyacrylamide gel (19:1; monomer/bis).

### Affinity cleaving titration fitting procedure

Gels were exposed to photostimulable storage phosphor imaging plates (Kodak Storage Phosphor Screen S0230 from Molecular Dynamics) at room temperature for 12–24 h. The data was transferred from the phosphor imaging screens to a Molecular Dynamics 400S PhosphorImager. Rectangles of identical dimensions were drawn around the cleavage bands at the target and the reference sites. The ImageQuant v.3.0 program running

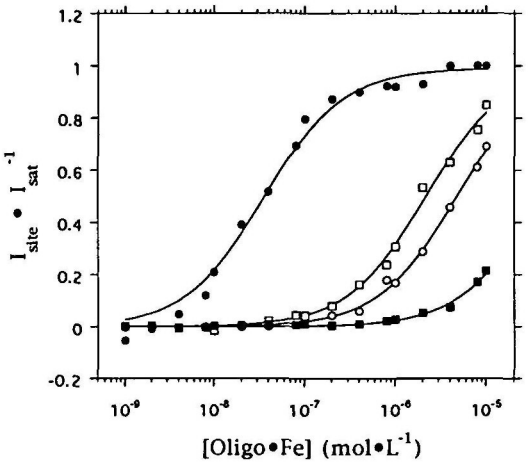


**Figure 6.** Data for quantitative affinity cleavage titration experiments from triple-helical complexes of oligodeoxyribonucleotide **5** containing the base analog F against all four Watson–Crick base pairs AT (●), CG (□), GC (○) and TA (■).

**Table 1.** Equilibrium association constants for either 15 mer **4** (Z=T) or **5** (Z=F) binding by triple-helix formation

| | $K_T$ [M$^{-1}$] [a] | |
|---|---|---|
| Watson-Crick base pair | Z = T | Z = F |
| AT | 5.0 (±0.4) x 10$^7$ | 2.4 (±0.6) x 10$^7$ |
| GC | 1.3 (±0.6) x 10$^5$ | 2.1 (±0.1) x 10$^5$ |
| CG | 1.4 (±0.1) x 10$^6$ | 4.0 (±1.4) x 10$^5$ |
| TA | < 10$^5$ | < 10$^5$ |

[a] Values reported in the table are mean values measured from affinity cleavage titration experiments performed in association buffer (10 mM Bis-Tris.HCl pH 7.0, 100 mM $Na^+$, 250 $\mu$M spermine tetrahydrochloride, 100 $\mu$M bp calf thymus DNA, 24°C)

on an AST Premium 386/33 computer was used to integrate the volume of each rectangle.

The affinity cleavage titration fitting procedure has been adapted from Singleton and Dervan (31, 32). The relative cleavage efficiencies at the target site for each oligodeoxyribonucleotide concentration were determined by using the following equation:

$$I_{site} = I_{tot} - \lambda I_{ref} \quad (6)$$

where $I_{site}$ and $I_{ref}$ are the intensities of the cleavage bands at the target and the reference site, respectively, and $\lambda$ is a scaling parameter defined as the ratio $I_{tot}/I_{ref}$ at the lowest oligodeoxyribonucleotide concentration. A theoretical binding curve, represented by eq.7 where $I_{sat}$ is the apparent maximum cleavage, $K_i$ the equilibrium association constant for oligonucleotide $I$, and $[O]_{tot}$ the oligodeoxyribonucleotide–EDTA concentration, was used to fit the experimental data using $I_{sat}$ and $K_i$ as adjustable parameters:

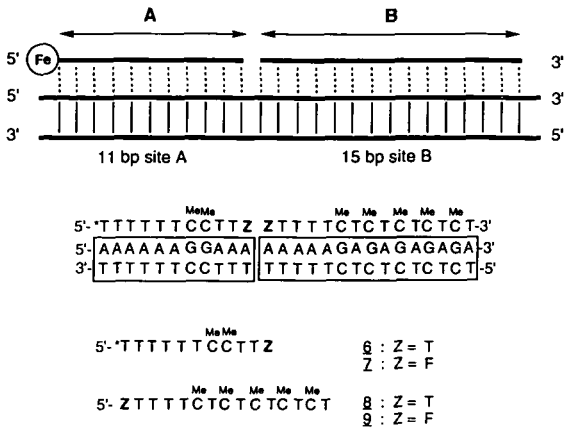


**Figure 7.** Schematic representation of a triple-helical complex containing two oligodeoxyribonucleotides bound at adjacent sites on double-helical DNA. Thick solid lines represent the DNA backbone and the associated oligodeoxyribonucleotides, thin solid or dashed lines indicate Watson–Crick or Hoogsteen hydrogen bonds.

$$I_{fit} = I_{sat} (K_i [O]_{tot}/1 + K_i [O]_{tot}) \quad (7)$$

Minimization of the difference between $I_{fit}$ and $I_{site}$ for all data points was performed using Kaleidagraph software. All values reported are the means of 3–6 experimental observations.

## RESULTS AND DISCUSSION

### Analysis of base specificity for triple helix formation

Five 15 mer oligodeoxyribonucleotides (Figure 4, **1**–**5**) that vary at one position Z, 5′-T*TT TTC TCT ZTC TCT- 3′ (Z = F, dA, dG, dC or T), and contain the DNA-cleaving moiety thymidine–EDTA·Fe (T*) at the 5′-end were synthesized by standard solid phase methods (26, 27). The pyrido[2,3-*d*]pyrimidine heterocycle F coupled with similar efficiency as the commercially available dA, dC, dG, and T phosphoramidites. The base composition of oligodeoxyribonucleotide **5** containing the novel base F was established by enzymatic degradation with snake venom phosphodiesterase and alkaline phosphatase, followed by HPLC analysis of the nucleoside monomers. To determine the relative affinities of the non-natural pyrido[2,3-*d*]pyrimidine heterocycle F for all four Watson–Crick base pairs within a pyrimidine–purine–pyrimidine triple-helix, the five 15 mer oligodeoxyribonucleotides-EDTA·Fe **1**–**5** were allowed to equilibrate for 24 h in association buffer (100 mM $Na^+$, 250 $\mu$M spermine tetrahydrochloride, 10 mM Bis-Tris–HCl, pH 7.0, 24°C) in the presence of the 232 bp DNA restriction fragments containing the target sequence with one variable base pair site (Figure 4B). The EDTA·Fe-mediated cleavage reactions were initiated by addition of dithiothreitol (DTT) and were allowed to proceed for 6 h. Separation and analysis of the cleavage products was performed by polyacrylamide gel electrophoresis. In addition to the known stable triplet combinations T·AT, C$^+$GC and G·TA, strong binding was observed for the combination Z = F, XY = AT.

To compare the association constants for the oligodeoxyribonucleotides **4** (Z = T) and **5** (Z = F) for all four Watson–Crick base pairs, the quantitative affinity cleavage titration method was used over an extensive concentration range using the same conditions as described above (Figures 5 and 6). The equilibrium association constant for the triplet combination Z = F, XY = AT was 2.4 $\times 10^7$ $M^{-1}$ (10 mM Bis-Tris–HCl pH 7.0, 100 mM $Na^+$, 250 $\mu$M spermine tetrahydrochloride, 100 $\mu$M bp calf thymus DNA), which is comparable in energy to Z = T, XY = AT (Figure 6, Table 1). Within a triple-helical complex F mimics T, suggesting that the predominant tautomeric form may be $F_2$. Alternatively, in order to accommodate the tautomeric form $F_1$, a backbone shift could occur to permit a wobble base in the third strand.

**Table 2.** Equilibrium association constants of either 11 mer **6** (Z=T) or **7** (Z=F) in the presence or absence of either 15 mer **8** (Z=T) or **9** (Z=F) bound to adjacent sites on DNA within a triple helical complex.

| oligo nucleotide | $K_T$ [$M^{-1}$][a] | Enhancement Factor | Stack (5′ - 3′) |
|---|---|---|---|
| **6** | 8.7 (± 2.6) x $10^5$ | | |
| **6** + **8** (1 μM) | 1.5 (± 0.1) x $10^7$ | 17 | TT |
| **6** + **9** (1 μM) | 5.4 (± 1.0) x $10^6$ | 6 | TF |
| **7** | 2.2 (± 0.6) x $10^5$ | | |
| **7** + **8** (1 μM) | 1.7 (± 0.6) x $10^6$ | 8 | FT |
| **7** + **9** (1 μM) | 7.2 (± 2.4) x $10^5$ | 3 | FF |

[a] Values reported in the table are mean values measured from affinity cleavage titration experiments performed in association buffer (25 mM Tris–acetate pH 7.0, 10mM $Na^+$, 1mM spermine tetrahydrachloride, 24°C).

### Analysis of base stacking

Substitution of the 5-position of 2′-deoxycytidines with a methyl group and 2′-deoxyuridines with a methyl-, ethynyl-, or 1-propynyl-group results in increased stability of triple-helical complexes (33–37). Similar effects have recently also been observed for two oligodeoxyribonucleotides binding adjacent triple-helix sites, presumably due to favorable energy of base stacking (38, 39). Quantitative affinity cleavage titration analysis was used to determine the cooperative interactions between an 11 mer and a 15 mer oligodeoxyribonucleotide bound to adjacent sites on DNA containing pyrido[2,3-*d*]pyrimidine F at the junction (Figure 7). Two 11 mers (**6** and **7**) containing T* at the 5′-end and either T or F at the 3′-end as well as two 15 mers (**8** and **9**) containing either T or F at the 5′-end were synthesized by standard solid phase methods. Either 11 mer **6** or **7** equipped with thymidine–EDTA at the 5′-position was allowed to equilibrate with a 272 bp 3′-$^{32}$P-end labeled duplex DNA restriction fragment containing the target sequence for 24 h in association buffer (10 mM $Na^+$, 1 mM spermine tetrahydrochloride, 25 mM Tris–acetate, pH 7.0, 24°C) in the presence or absence of either 15 mer **8** or **9** at $\mu$M concentrations sufficient to saturate the adjacent 15 base pair target site. From affinity cleavage titration analysis, a 17-fold increase in equilibrium association constant was observed when oligodeoxyribonucleotide **6** bound in the presence of **8** (5′-TT-3′ stack) (38). However, binding enhancements of only 8 and 6 were observed when

oligodeoxyribonucleotide **7** bound in the presence of **8** (5′-FT-3′ stack) and when oligodeoxyribonucleotide **6** bound in the presence of **9** (5′-TF-3′ stack). Only a 3-fold enhancement was observed if both oligodeoxyribonucleotides contained terminal pyrido[2,3-*d*]pyrimidines at the junction (**7** in the presence of **9**) (Table 2). These observations indicate that modification of pyrimidine nucleosides to form extended ring systems does not necessarily result in increased favorable base stacking interactions.

## CONCLUSION

In summary, within double-helical DNA F pairs with G > A, whereas within a pyrimidine Hoogsteen strand of triple-helical complexes F pairs with A. The simplest explanation of our data is that the pyrido[2,3-*d*]pyrimidine F appears to bind single and double stranded DNA in various tautomeric forms. However, we cannot rule out other hydrogen bonding pairing schemes which would require backbone deformations. Clearly, direct structural studies such as 2-D NMR will be required to confirm the existence of different tautomers of F within double-helical and triple-helical nucleic acids.

## ACKNOWLEDGEMENTS

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